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Ligand Based Pharmacophore Identification and Molecular Docking Studies for Grb2 Inhibitors

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Grb2 is an adapter protein involved in the signal transduction and cell communication. The Grb2 is responsible for initiation of kinase signaling by Ras activation which leads to the modification in transcription. Ligand based pharmacophore approach was applied to built the suitable pharmacophore model for Grb2. The best pharmacophore model was selected based on the statistical values and then validated by Fischer's randomization method and test set. Hypo1 was selected as a best pharmacophore model based on its statistical values like high cost difference (182.22), lowest RMSD (1.273), and total cost (80.68). It contains four chemical features, one hydrogen bond acceptor (HBA), two hydrophobic (HY), and one ring aromatic (RA). Fischer's randomization results also shows that Hypo1 have a 95% significant level. The correlation coefficient of test set was 0.97 which was close to the training set value (0.94). Thus Hypo1 was used for virtual screening to find the potent inhibitors from various chemical databases. The screened compounds were filtered by Lipinski's rule of five, ADMET and subjected to molecular docking studies. Totally, 11 compounds were selected as a best potent leads from docking studies based on the consensus scoring function and critical interactions with the amino acids in Grb2 active site.

Key Words : Growth factor receptor-bound protein 2, Son of sevenless, Pharmacophore, Hypogen, ADMET, Molecular docking

Introduction

Growth factor receptor-bound protein 2 (Grb2) is a 25 kDa protein,[1] plays an important role between a phosphotyrosine signal and downstream cellular events as an adaptor protein.[2-4] Grb2 is widely expressed in epithelial cell growth, encoded by *grb2* gene. Grb2 is involved in the signal transduction pathway and essential for multiple cellular functions. It regulates the Ras activation through its association with guanine nucleotide exchange factor of SOS and initiates the MAP kinase pathway which leads to many cancers.[5-7] Grb2 has linked with an epidermal growth factor receptor tyrosine kinase to activate Ras, Erk1 and Erk2 (Extracellular Signal-Regulated Kinases).[8-11] It is also important for linking receptor tyrosine kinases to small GTP-binding protein signaling, such as growth factor-induced cytoskeleton organization.[12,13] The Grb2-SOS complex can bind with insulin receptor substrate-1 (IRS-1) which is one of the primary targets for insulin and insulin-like growth factor receptors. Furthermore, the independent of IRS-1 and Grb2 links the insulin receptor to Ras signaling through another Shc adapter protein.[14] Grb2, emerged as a potent therapeutic target for anticancer therapy, play a vital role in morphogenesis as well as angiogenesis. Many researchers reported that the small molecules which can inhibit of the Grb2 function could be a potential anticancer agent by block the transformation and proliferation of various cell types.

There are many reported inhibitors available to bind in the SH2 domain of Grb2 to inhibit its function. Moreover, protein tyrosine kinase (PTK) inhibitors such as Gleevec and CEP-701 are necessary to stop the Grb2 function. The $P27^{Kip1}$ is a downregulated in aggressive human cancers and this (P27) inhibitor can inhibit Grb2 function by blocking its association with guanine nucleotide exchange factor of SOS[16] and these can be important information for the development of anti-cancer agents.[17]

In this work pharmacophore modeling and molecular docking approaches has been employed to identify the small molecules which contain the important chemical features to inhibit the function of Grb2. The HypoGen algorithm was used to develop the 3D pharmacophore models based on the diverse set of experimentally proved Grb2 inhibitors. The best pharmacophore model was selected based on its statistical values and validated by Fischer's randomization method and test set. The validated best pharmacophore hypothesis was used as a 3D query to search the various chemical databases, namely Maybridge, Chembridge, and NCI2000. Drug-like compounds with predicted pharmacophoric features along with the good estimated activity values were retrieved from the databases and evaluated using molecular docking studies.

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Materials and Methods

Preparation of Molecules. Pharmacophore modeling is an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target. We have collected 38 inhibitors, tested using same biological assay method from scientific literature.[18] The 2D formats of all molecules were produced using Chemsketch[19] and converted into 3D structures using Discovery Studio (DS).[20]

Preparation of Training and Test Sets. The best training set molecules should satisfy the certain criteria's like it cover wide range of activity values which span four orders of magnitude and contain a minimum of 16 structurally diverse molecules.[21] The test set was used to validate the hypothesis whether it can able to predict the activity values of diverse compounds beyond the training set in the same order of magnitude or not. The CHARMm force filed was applied to each molecule in the training and test sets and 255 conformations were generated for each compound using *Best conformational* model with an energy constraint of 20 kcal/mol.[22] The *Feature Mapping* method[23] was used to select the important chemical features present in known highly active Grb2 inhibitors.

Database Search and ADMET Calculation. Virtual screening technique was used to prioritizing chemical compounds for identifying hit compounds from database. Finding the drug-like properties from virtual screening is an important process for further *in vitro* studies.[24,25] *Ligand pharmacophore mapping* protocol was used for virtual screening by applying *Fast/Flexible* search option implemented in DS.

The screened compounds from database search were filtered by applying maximum fit value, Lipinski's rule of five[26] and ADMET properties (Adsorption, Distribution, Metabolism, Excretion and Toxicity). The Lipinski's rule of five was used to evaluate the drug-likeness or determine whether a chemical compound has certain pharmacological property to be an orally active drug in humans.[27] The criteria for Lipinski rule of 5 (a) less than 5 hydrogen bond donors, (b) not more than 10 hydrogen bond acceptors (c) molecular weight should be under 500 Daltons, and (d) LogP should be less than 5. The ADMET describes the important pharmacokinetic properties of a drug which will be more helpful to find the orally bioactive compound. The hit compounds were sorted based on the above rules and subjected to molecular docking studies.

Molecular Docking. The molecular docking has become a powerful method to predict or increase the efficiency in lead optimization. The docking based pharmacophore model was used to find the critical interaction between protein and ligand. Molecular docking elucidates how well the small molecules interact with protein.[28] There are many Grb2 complex crystal structures available in Protein Data Bank (PDB, www.rcsb.org).[29] Based on the resolution, the crystal structure of Grb2-SH2 domain complex with a flexible Ac-pY-E-N-NH2 tripeptide mimic (PDB ID: 3KFJ) was selected as a receptor. The water molecules were removed and the hydrogen atoms were added by applying CHARMm force field using DS. *LigandFit* module was used to dock the small molecules into the active site of Grb2. *LigandFit* performs docking in three stages such as docking, *in-situ* ligand minimization, and scoring. During docking, an attempt is made to dock a ligand or series of ligands into a user defined binding site. If a receptor molecule is specified for ligand minimization, the minimization will perform in the presence of the receptor otherwise ligands are minimized in vacuum and also the receptor is held rigid. In scoring stage the generated ligand poses were scored for a strong binding at the active site.

Define and Edit Binding Site/DS was used to identify receptor binding site for a ligand molecule and Smart Minimizer algorithm was used to minimize the protein. All docking parameters were set at their default values. Ligscore and Piecewise Liner Potential (PLP), Potential Mean Force (PMF), Jain scoring[30] and Ludi[31,32] scoring functions were used to find ligand binding affinity. Top ten conformations were generated for each ligands based on its dock score.

Results and Discussion

Pharmacophore Generation. Totally 38 compounds were collected from the literatures[33] among them 16 were selected as training set and remaining compounds as test set. Structures and biological activities of the training set compounds are shown (Figure 1). The training set compounds includes nonphosphorylated cyclopeptide, a variety of potent nonphosphorylated cyclopeptide (Grb2-SH2 domain antagonists and indole-3-yl propylamine derivatives) inhibitors of scaffolds which resulted in high affinity to Grb2-SH2 domain. These available compounds provide ideal chemical structures for the development of Grb-SH2 domain inhibitors. The maximum number of 255 conformations was generated for each molecule using Poling algorithm with a constraint of 20 kcal/mol energy cutoff value above the global minimum.

The *Feature Mapping* protocol contains various chemical features but only Hydrogen Bond Acceptor (HBA), Hydrogen Bond Donor (HBD), and Hydrophobic (HY) features were mapped well with most of the highly active compounds in training set. Thus, these chemical features were used to generate the hypotheses based on the activity value of training set compounds. The generated top ten hypotheses contain the combination of three chemical features such as HBA, RA, and HY. The statistical values of the best ten pharmacophore hypotheses have shown (Table 1). Debnath's analysis was used to select the best hypothesis among ten hypotheses. The fixed cost is the sum of cost components that includes weight cost, error cost and configuration cost and represents a cost of the theoretical ideal hypothesis. This could absolutely predict the activity of compounds in the training set with lowest deviation, while null cost represented the cost of hypothesis with no features that estimates every activity to be the average activity. The fixed and null cost values are 63.51 and 262.91, respectively. A value of

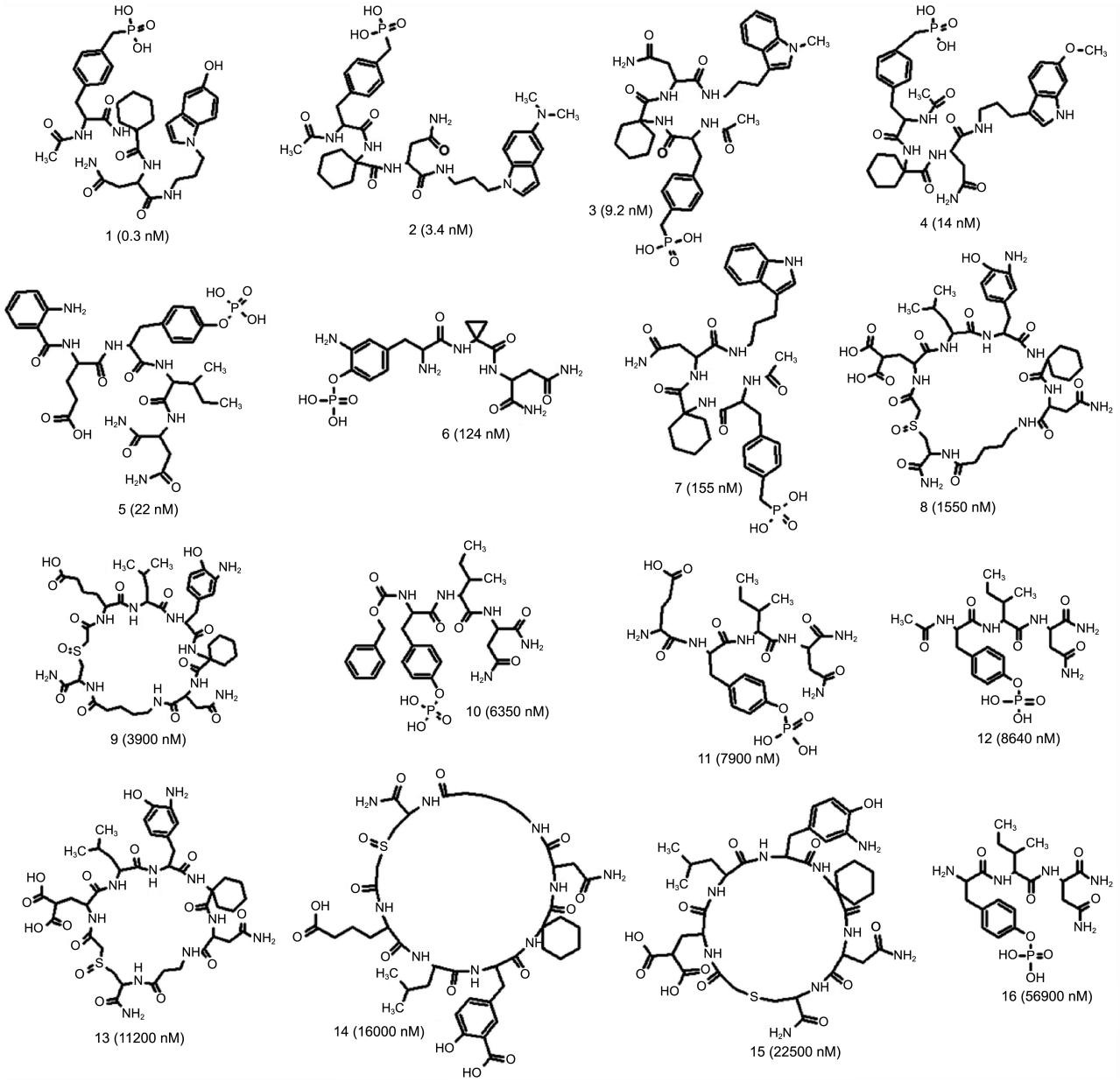

Figure 1. 2D structures of training set compounds. The compound numbers and IC_{50} values are shown at the bottom of compounds.

Table 1. Statistical values of the top ten pharmacophore hypotheses

Name	Total cost	Cost difference[a]	RMS[b]	Correlation	Features[c]			Max fit
					HBA	RA	HY	
Hypo1	80.68	182.22	1.27	0.97	1	1	2	11.49
Hypo2	91.72	171.19	1.80	0.93	1	1	2	10.56
Hypo3	94.12	168.79	1.94	0.92	1	1	2	08.76
Hypo4	94.82	168.09	1.95	0.92	1	1	2	09.40
Hypo5	95.87	167.04	1.98	0.92	1	1	2	09.50
Hypo6	97.25	165.66	2.04	0.91	1	1	2	09.06
Hupo7	99.12	163.79	2.08	0.91	1	1	2	09.45
Hupo8	99.69	163.22	1.98	0.92	1	1	2	11.66
Hypo9	100.20	162.71	2.09	0.91	1	1	3	12.56
Hypo10	100.69	162.22	2.13	0.91	1	1	2	09.58

The Null cost value is 262.91; Fixed Cost is 63.51; Configuration Cost is 15.94. [a]The difference between Null Cost and Total Cost. [b]RMS-Root Mean Square. [c]HBA-Hydrogen Bond Acceptor; RA-Ring Aromatic; HY-Hydrophobic.

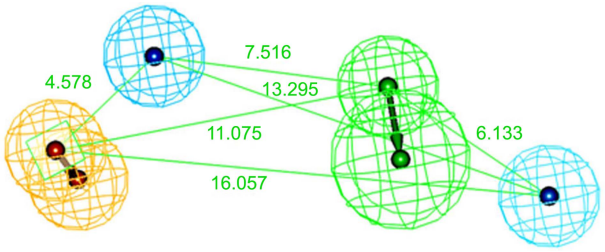

Figure 2. Pharmacophore was generated (Hypo1) using 3D QSAR Pharmacophore generation module. (a) hydrogen bond acceptor (HBA, green), ring aromatic (RA, orange) and hydrophobic (HY, cyan) features. (b) Hypo1 is shown with distance constraints.

40-60 bit cost difference indicates that the hypothesis to show over 90% statistical significance but Hypo1 shows the cost difference of greater than 180 bits indicating its true correlation data. Hypo1 shows a good statistical value such as the lowest error value of 59.43, highest cost difference of 182.22, high maximum fit value of 11, and lowest RMSD value of 1.2. Hence Hypo1 was selected as a best hypothesis which consists of one hydrogen bond acceptor (HBA), two hydrophobic (HY), one ring aromatic (RA). The chemical features and geometric parameters of Hypo1 are shown (Figure 2).

The training set molecules were classified into three categories based on their activity values such as highly active ($IC_{50} \leq 150$ nM), moderately active (150 nM > IC_{50} < 15,000 nM), and less active ($IC_{50} \geq 15{,}000$ nM). One active compound in the training set was underestimated as moderately active, one and three moderately active compounds were

Table 2. Experimental and predicted activity values of the training set molecules based on the pharmacophore model of Hypo1

Compound No	Fit Value	Exp. IC_{50} nM	Pred. IC_{50} nM	Error[a]	Exp. Scale[b]	Pred. Scale[b]
01	10.24	0.3	0.39	+1.3	+++	+++
02	9.30	3.4	3.4	-1.0	+++	+++
03	8.93	9.2	8	-1.1	+++	+++
04	8.43	14	25	+1.8	+++	+++
05	7.80	22	110	+4.9	+++	+++
06	7.58	124	180	+1.4	+++	++
07	7.87	155	91	-1.7	++	+++
08	6.47	1550	2300	+1.5	++	++
09	5.74	3900	12000	+3.1	++	++
10	6.95	6350	770	-8.3	++	++
11	5.75	7900	12000	+1.5	++	+
12	5.75	8640	12000	+1.4	++	+
13	5.75	11200	12000	+1.1	++	+
14	5.75	16000	12000	-1.3	+	+
15	5.74	22500	12000	-1.8	+	+
16	5.75	569000	12000	-4.7	+	+

[a]'+' indicates that the experimental activity value is lower than the predicted IC_{50} value. '–' indicates that the experimental activity value is higher than the predicted IC_{50} value. [b]Activity scale: $IC_{50} \leq 150$ nM = +++ (highly active); 150 nM > IC_{50} < 15,000 nM = ++ (moderately active); $IC_{50} \geq 15{,}000$ nM = + (low active).

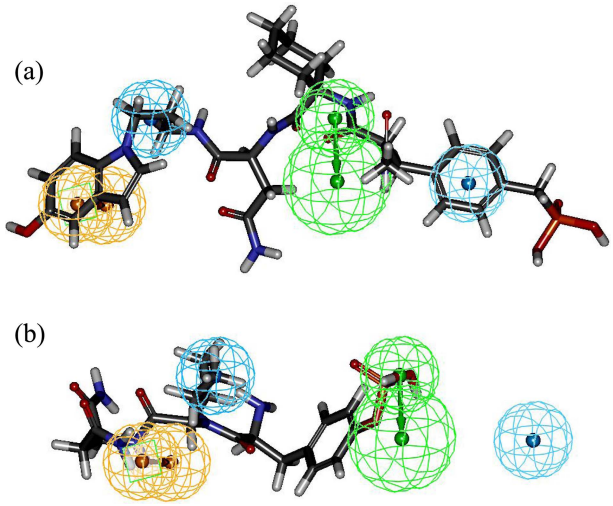

Figure 3. (a) Best pharmacophore model Hypo1 aligned with most active compound (Compound1 IC_{50}: 0.3 nM) and (b) less active compound (Compound16 IC_{50}: 56900 nM) was overlaid upon Hypo1.

overestimated as active and underestimated as less active compounds by Hypo1, respectively (Table 2). All the remaining compounds in the training set were estimated in their activity scale by Hypo1. The most active compounds of training set (Compound1 IC_{50}: 0.3 nM) and their features are overlaid upon Hypo1 but less active compound (Compound 16 IC_{50}: 56900 nM) not fitted well with Hypo1. The most active and less active compounds in the training set were aligned in Hypo1 was shown (Figure 3).

Pharmacophore Validation.

Fischer's Randomization Method: Fischer's randomization method is used to assess the quality of the Hypo1 pharmacophore model[34] by randomly reassign the activity values of molecules in the training set and these scramble spread sheets are used to generate the new hypotheses. The aim of this validation is to check robustness of the Hypo1 model. In this validation, to achieve 95% confidence level, 19 spreadsheets were generated by shuffling the active value

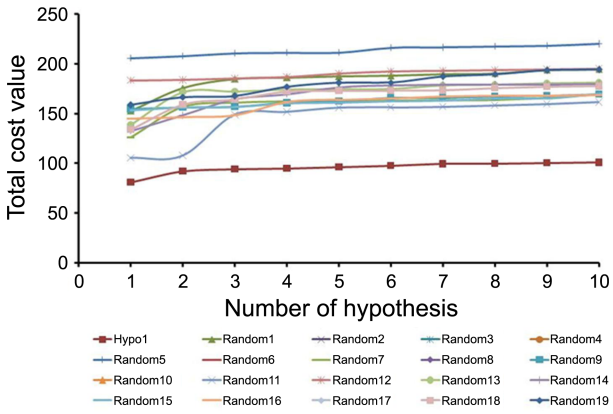

Figure 4. Fischer's randomization result. 13 random spread sheets were generated from 19 random spread sheet generations. Number of hypothesis is shown in x axis and total cost value showed in y axis.

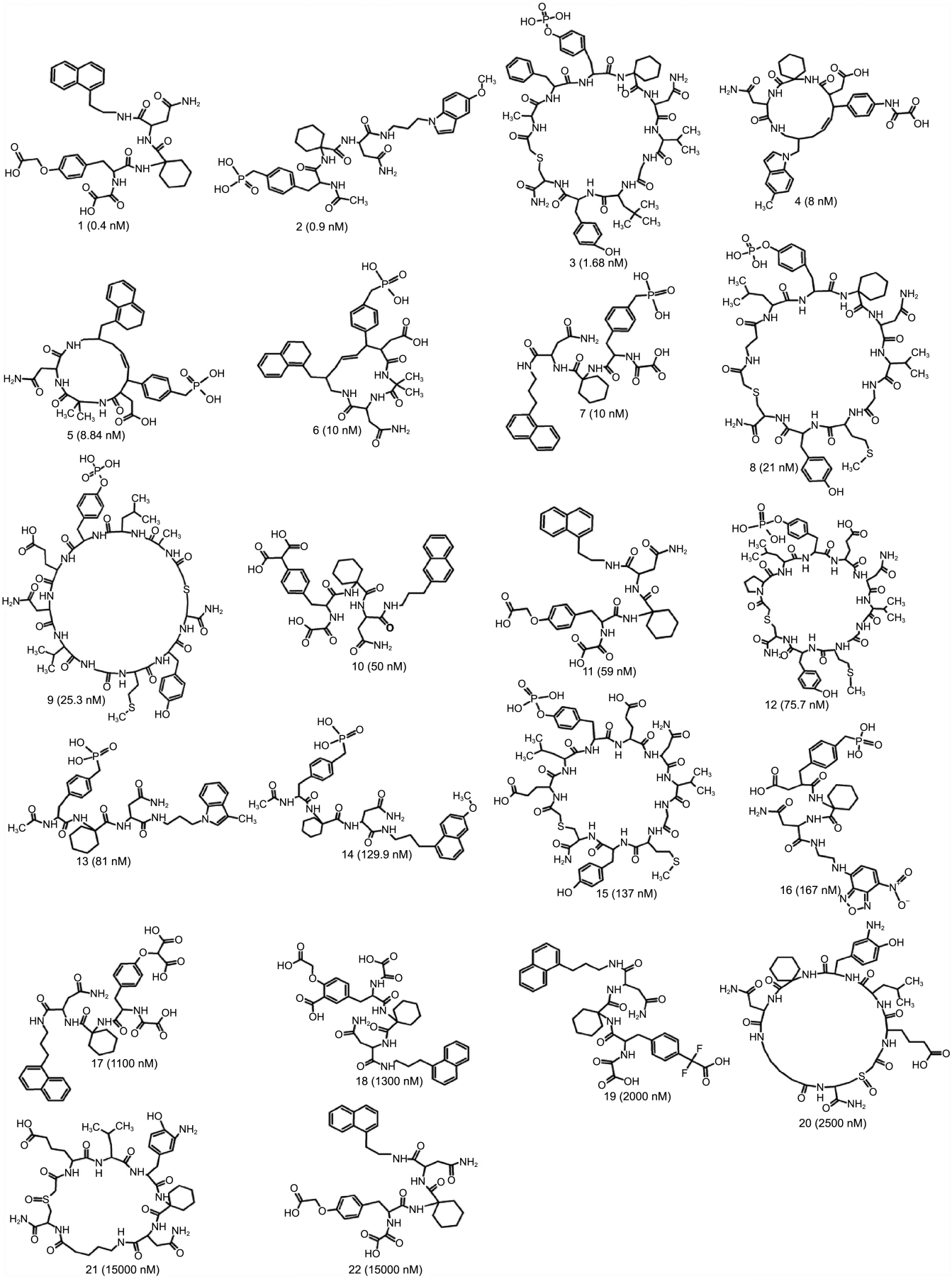

Figure 5. 2D structures of test set compounds. The compound numbers and IC_{50} values are shown at the bottom of compounds.

Table 3. Experimental and predicted activity values of 22 test set molecules against Hypo1

Compound No	Fit Value	Exp. IC_{50} nM	Pred. IC_{50} nM	Error[a]	Exp. Scale[b]	Pred. Scale[b]
01	10.22	0.4	0.71	1.78	+++	+++
02	09.89	0.9	1.57	1.75	+++	+++
03	09.54	1.68	1.94	1.15	+++	+++
04	08.89	8	10.47	1.30	+++	+++
05	08.74	8.84	12.26	1.46	+++	+++
06	08.45	10	23.81	2.38	+++	+++
07	08.45	10	23.81	2.38	+++	+++
08	08.68	21	14.11	1.48	+++	+++
09	08.31	25.3	43.31	1.71	+++	+++
10	08.01	50	63.23	1.32	+++	+++
11	04.94	59	99.00	1.67	+++	+++
12	08.01	75.7	36.96	2.04	+++	+++
13	07.84	81	98.54	1.21	+++	+++
14	07.41	129.9	266.33	2.05	+++	++
15	07.83	137	100.52	1.36	+++	+++
16	07.58	167	476.41	2.85	++	++
17	06.83	1100	2000.57	1.81	++	++
18	06.48	1300	2230.29	1.71	++	++
19	06.75	2000	1195.88	1.67	++	++
20	06.70	2500	1000.53	2.49	++	++
21	05.70	15000	13384.10	1.12	+	+
22	05.58	15000	17681.40	1.17	+	+

[a]Error value shown in all positive values, it's directly related to the experimental and predicted activity value. [b]Activity scale: Experimental activity scale +++, $IC_{50} \leq 150$ nM is highly active; ++, 150 nM > IC_{50} < 15,000 nM are moderate active; +, $IC_{50} \geq 15{,}000$ nM are less active.

of the compounds present in the training set using following formula $[1-(1+X)/Y] \times 100$, where X, total number of hypotheses having a total cost lower that Hypo X and Y, total number of Hypogen runs (initial + random runs). Here, X = 0 and Y = (19 + 1), S = $[1-(1+0)/(19+1))] \times 100\% = 95\%$. Out of 19 random spread sheets, 13 spread sheets could produce the hypothesis but remaining 6 spread sheets were failed to generate the hypothesis (Figure 4). However, the total cost values of all scrambled hypotheses were greater than the original hypothesis which proved the robustness of Hypo1 hypothesis.

Test Set: The test set, consist of 22 structurally distinct compounds, was used to check whether Hypo1 can able to predict the activity of compounds in same order of magnitude other than the training set or not. The test compounds (Figure 5) were classified into three sets based on their activity scale: $IC_{50} \leq 150$ nM: highly active (+++), 150 nM > IC_{50} < 15,000 nM: moderate active (++), $IC_{50} \geq 15{,}000$ nM: less active (+). Hypo1 underestimated the one active compound as moderately active (Compound. No 14) and all the remaining compounds were predicted in their own activity scales (Table 3). Moreover, error values of all compounds in the test set was less than 2.5 and the correlation of coefficient value for the test set is 0.94. This test validation indicates that the Hypo1 can able to predict the compounds in their own activity range other than the training set compounds. Hence, the best pharmacophore model (Hypo1) was used as a query in virtual screening process.

Table 4. The fit value and their predicted IC_{50} value for the final hit compounds from virtual screening

Compound No	Compound Name	Fit Value[a]	Pred. IC_{50} nM[b]
01	NCI0169143	11.25	0.03
02	NCI0029868	11.27	0.03
03	NCI0643540	11.22	0.04
04	NCI0613586	11.21	0.04
05	NCI0644964	11.08	0.05
06	NCI0029866	11.12	0.05
07	NCI0668890	11.08	0.05
08	NCI0667653	11.07	0.05
09	NCI0243544	11.06	0.05
10	NCI0055732	11.06	0.05
11	NCI0164083	11.04	0.06

[a]Fit value of 11 database compounds have been shown in the ranges between 0.03-0.06. [b]Predicted activity values (11.04-11.27) of the database compounds were shown.

Database Search. The main purpose of virtual screening is to find a novel scaffold to inhibit the activity of various targets.[35,36] The mode of action is to finding new molecules from the database for biological testing. Hypo1 was used as a 3D structural query for retrieving potent Grb2 leads from three chemical databases including Maybridge (60,000 compounds), Chembridge (50,000 compounds), and NCI2000 (239,000 compounds). There are 3,087 molecules from Maybridge, 1,477 molecules for Chembridge and 6,326 compounds from NCI2000 have been satisfied all chemical features present in the Hypo1. Totally, 10,890 hit compounds were sorted out to 482 compounds by applying maximum fit value of 11. The sorted 482 molecules (Maybridge (23), Chembridge (23), NCI2000 (436)) were tested for Drug-like and ADMET properties. Finally, 11 compounds were selected based on the above criteria that includes the fit value of 11.02 to 11.27 (Table 4) and subjected to molecular docking studies for further refinement.

Molecular Docking. The main aim of docking study is to find the binding affinity between protein-ligand complexes. Training set compounds (Compound1 IC_{50}: 0.3 nM) and 11 hit compounds retrieved from the database screening which satisfied the drug-like properties were docked in the active site of Grb2 using *LigandFit*[38] module in DS and top ten poses were saved for each molecule. In the case of training set molecules, most of the active compounds show an average fitness scores more than 7 and dock score of 100.

The active training set compounds showed hydrogen bond and hydrophobic interactions with active site residues of Grb2 such as Arg67, Arg86, Ser90, Ser96, Lys109, and His107. The nitro group in inhibitor forms a salt bridge with Lys109 and some of the active compounds in the training set show strong hydrogen bond interaction with Ser90 and Ser96. The overall interaction between the inhibitor and the receptor is not weakening because the carboxyl group makes extensive hydrogen bond interactions with Arg67 and Arg86.

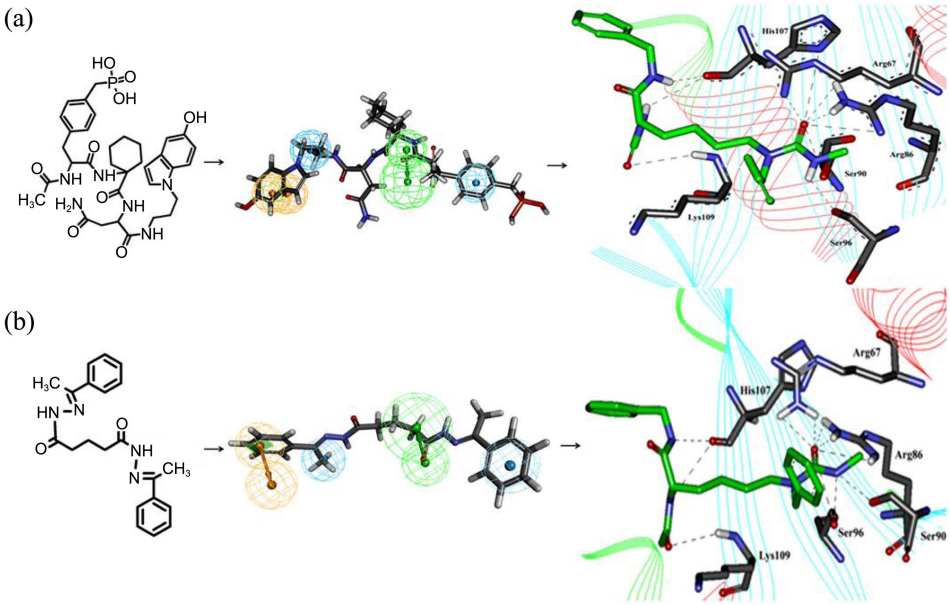

Figure 6. (a) Docking result of training set compound. The docked confirmation of training set compound (Compound1 IC_{50}: 0.3 nM) shows crucial interaction with nitro and carboxy groups of active site residues in Grb2 protein (Pdb ID: 3KFJ). Hydrogen bonds are shown in black dotted lines and (b) Docking result of database compound. The binding modes of the database screened compound (NCI0169143) shows strong interaction with active site residues of Grb2 protein (Pdb ID: 3KFJ). Hydrogen bonds are shown in black dotted lines.

In addition, database hit compounds formed hydrogen bond and hydrophobic interactions with most of the critical residues such as Arg67, Arg86, Ser90, Ser96, Lys109, and His107 and fitness scores of 11 and good dock score values of above 100. The 2D structures of active training set compound (Compound1 IC_{50}: 0.3 nM) and Hypo1 overlay with active compound and binding modes of the active compound with active site residues of Grb2 have shown (Figure 6(a)). The 2D structures of final hit compound (NCI0169143) and Hypo1 overlay with hit compound and binding modes of the hits with active site residues of Grb2 and most interaction have shown (Figure 6(b)).

Conclusions

The aim of this pharmacophore modeling based molecular docking is to find a new leads from database screening to inhibit the function of Grb2. We have implemented a ligand-based pharmacophore modeling to identify the vital chemical features to inhibit Grb2 activity. The best pharmacophore model was developed for Grb2 based on the currently available inhibitors. The Hypo1, best pharmacophore model consists of four chemical features: one HBA, one RA, and two HY, it shows a good cost difference of 182.22, lowest RMSD (1.2), and total cost (80.68). The correlation coefficients of training set and test set were 0.97 and 0.94, respectively. The Fischer's randomization results have clearly shown that 95% strong confidence on an accurate and reasonable pharmacophore model Hypo1 with statistical significance and it is not generated by chance. Hypo1 was used as a 3D query for screening large databases like Maybridge, Chembridge, and NCI2000. Totally, 11 drug-like hit compounds were selected for molecular docking studies which satisfied all the chemical features of Hypo1, shows good fit value, ADMET, and Lipinski's rule of five. All the hit molecules have shown high dock score (above 100) and formed hydrogen bond and hydrophobic interactions with the most of the critical residues in Grb2. Based on the above validations we suggest that the chemical feature of Hypo1 is important for the development of Grb2 inhibitor.

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